

UNITED STATES PATENT AND TRADEMARK OFFICE ANNEX U.S. 111

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That I am knowledgeable in the English language and in the language in which the below-identified international application was filed, and that I believe the English translation of the international application PCT/EP01/01663 is a true and complete translation of the above-identified international application as filed.

I hereby declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under section 1001 of Title 18 of the United States Code and that such willful false statements may jeopardize the validity of the application or any patent issued thereon.

Date October 1, 2001

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GK-ZEI-3140METHOD AND SYSTEM FOR DETECTING THE LIGHT COMING FROM A
5 SAMPLE

Fluorescence correlation spectroscopy (FCS) implemented in a microscope construction (FCM) has proven successful for investigating biomolecular interactions particularly where the investigations are carried out¹ in very small ranges of concentration of less than 1 μmol and in measurement volumes of less than 10^{-14}l . The measurement location plays only a minor role, provided the specimens to be examined are homogeneous. However, in connection with structured specimens such as biological cells, knowledge and selection of the measurement location is critically important. Formerly, this knowledge of the measurement location was gained by conventional transmitted-light and incident-light microscopy. For this purpose, switching was carried out between the FCS detection unit and a conventional fluorescence microscope arrangement. The use of conventional microscopy has several disadvantages. On the one hand, the specimens are exposed to high radiation loading; on the other hand, the optimal measurement location can not be localized in three-dimensional coordinate systems with the required accuracy of less than 1 μm .

The arrangements and methods described in the following in the patent claims and with reference to the drawings advantageously make it possible to expand the FCS method to an imaging method (S-FCM). In this way, information can be gained concerning the spatial distribution of the molecular interactions under investigation.

Figure 1 shows a first advantageous arrangement. With a microscope unit MU (in this case, an inverted microscope for observing a specimen P via an objective O arranged below the specimen and a tube lens TL, the specimen P being located on a table T which is displaceable in x-, y- and z-direction), light from a laser light source LLS with one or more wavelengths is focused by a scanning unit SU in a specimen either directly or through a light-conducting fiber LF via collimating optics KO and a primary beam splitter STPS. The scanner S allows the

light beam to be deflected in x-direction and y-direction; different specimen layers can be detected through vertical adjustment of the specimen table T or of objective O. The light coming from the specimen passes through the scanner S again and is assigned to detection channels DES1...N by means of the secondary beam splitter STSS 1...N and converted into electrical signals for evaluation via a control unit CU in a computer. The measured signals are used to obtain image information. By means of a beam switching unit BS, e.g., of a fully reflecting mirror or partially transmitting mirror which can be swiveled in and out, light LLF from a laser light source with one or more wavelengths is focused in the specimen by means of an FCS unit FU via a primary beam splitter STPF.

The light sources LLS and LLF may also be identical and can be coupled into the units SU and FU by suitable deflecting and switching elements. The fluorescent light coming from the specimen is directed through secondary beam splitters STSF 1...N into one or more FCS detection channels DEF 1...N and, for purposes of evaluation, converted into electrical signals and sent to the control unit CU. The signals are used for FCS analysis.

Autocorrelation analyses or cross-correlation analyses can be carried out depending on the detection channels that are installed. In this connection, for example, diffusion times, particle numbers, lifetimes and proportions of components are determined at the installed detection channels.

Data acquisition is controlled for both detection units by the same control unit CU and a computer C with a suitable program. Control of the specimen table T, vertical adjustment of the objective O and beam switching unit BS is also carried out by this computer-controlled control unit. Accordingly, as a result of integrating an FCS detection unit in a confocal laser scanning microscope system, it is also possible to combine FCS analysis results of measurements at various specimen locations to form images. This results in an advantageous arrangement which is suitable for determining FCS measurement locations with great accuracy while protecting the specimen and also for using FCS analysis results of measurements at different locations for generating images.

It is advantageously possible, for example, to use different colors to generate a color two-dimensional or three-dimensional depiction of diffusion times or other analysis results, depending on the measurement location.

5 Further, through the use of storage allocation, the recorded FCS image can be combined graphically, e.g., as an added color, with LSM images of different colors per channel.

FCS/LSM differentiating elements or quotient-forming elements or other combinations can also be formed and represented.

10 Modification, according to the invention, of the laser scanning microscope and operative combination with the FCS device unit are carried out by suitable programs in the computer by means of a device control unit shared by all of the components. The scanning unit, FCS unit, microscope unit and specimen position system are mechanically, optically and electronically adapted to one another and combined.

15 After the specimen is scanned, image points can be marked, brought into the measurement position for the FCS unit and measured.

The relevant points can be selected automatically according to given criteria (e.g., raster, structure detection of the image) or by the user according to individual assessment of the image recorded by the scanning unit.

20 The suggested construction makes possible a deliberate selection of microscopically small measurement locations in the specimen to be investigated by the FCS method. Further, it is possible to graphically detect and depict the spatial variation of the FCS analysis results from a systematic sequence of FCS measurements images and to correlate them with the LSM image.

25 Figure 2 shows another advantageous arrangement. In this case, a shared laser unit LLSF and a shared primary beam splitter STPFS are provided in or at a shared unit SFU. Separate beam splitters and detectors DEF, STSF and DES, STSS are shown for LSM detection and FCS detection. The detectors DEF and DES can advantageously be identically constructed. The measurement mode and
30 evaluating mode can be selected by means of the control unit CU. The common localization of the LSM and FCS detection channels in one unit results in an advantageous arrangement. As in the construction which was described with

reference to Figure 1, laser light of one or more wavelengths is focused through a scanning unit in the specimen by means of collimating optics and the primary beam splitter. A special advantage of this construction is that the measurement location for FCS measurement can also be selected by means of the scanner. In particular, it is possible in this way to advantageously expand the FCS analysis method to an imaging method (scanning FCM: S-FCSM). Reflected light and fluorescent light are captured by the objective, pass again through the scanner and are deflected by one or more secondary beam splitters into one or more LSM detection channels or FCS detection channels. Separation can then be carried out according to spectral characteristics or polarization characteristics. The detected light induces electrical signals which are directed to the control unit connected to the computer, including a suitable program, and are used in the latter for FCS analysis or image reconstruction.

In this case, the laser scanning microscope is modified in such a way that it contains components and evaluating procedures which also make it possible to carry out FCS measurements. Scan components and FCS components are combined, according to the invention, in such a way that beam splitting or beam switching such as is shown in Figure 1 can be dispensed with. The advantage of this arrangement consists in that no specimen movements are necessary for carrying out the FCS analyses at the previously selected points because the measurement location can be adjusted by means of the scanner and through vertical adjustment of the objective.

The effective connection of the operating modes is carried out in that either the scanner is stopped directly during the scanning process and an FCS evaluation is carried out at the specimen points set in this way or, after the scanning process, an FCS evaluation is carried out by adjusting the mirrors or displacing the table while the scanner mirrors are stopped at determined points. The arrangement according to Figure 2 enables FCS measurements with high positioning accuracy in quick succession. This suggested construction comprises a scanning fluorescence correlation microscope (S-FCM) which can show structural as well as biochemical information in images according to the invention.